PMP1 18-38, A Yeast Plasma Membrane Protein Fragment, Binds Phosphatidylserine from Bilayer Mixtures with Phosphatidylcholine: A ^{2}H-NMR Study

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ABSTRACT PMP1 is a 38-residue plasma membrane protein of the yeast *Saccharomyces cerevisiae* that regulates the activity of the H^+-ATPase. The cytoplasmic domain conformation results in a specific interfacial distribution of five basic side chains, thought to strongly interact with anionic phospholipids. We have used the PMP1 18-38 fragment to carry out a deuterium nuclear magnetic resonance (^{2}H-NMR) study for investigating the interactions between the PMP1 cytoplasmic domain and phosphatidylserines. For this purpose, mixed bilayers of 1-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) were used as model membranes (POPC/POPS 5:1, m/m). Spectra of headgroup- and chain-deuterated POPC and POPS phospholipids, POPC-d4, POPC-d31, POPS-d3, and POPS-d31, were recorded at different temperatures and for various concentrations of the PMP1 fragment. Data obtained from POPS deuterons revealed the formation of specific peptide-POPS complexes giving rise to a slow exchange between free and bound PS lipids, scarcely observed in solid-state NMR studies of lipid-peptide/protein interactions. The stoichiometry of the complex (8 POPS per peptide) was determined and its significance is discussed. The data obtained with headgroup-deuterated POPC were rationalized with a model that integrates the electrostatic perturbation induced by the cationic peptide on the negatively charged membrane interface, and a "spacer" effect due to the intercalation of POPS/PMP1f complexes between choline headgroups.

INTRODUCTION

It is now well established that specific lipid-protein interactions are involved in numerous biochemical events associated with membrane functions. In particular, the role of anionic phospholipids in protein insertion and translocation (Van Klompenburg and de Kruijff, 1998; Von Heijne, 1992) and in signaling pathways (Newton, 1998; Zwaal et al., 1998) has been extensively investigated. Specific lipid-protein association also leads to lateral domain formation whose biological role is now better understood (Kurzchalia and Parton, 1999; Sabra and Mouritsen, 1998). However, the molecular mechanisms that govern the specificity of lipid-protein interactions are far from be fully characterized. This is partly due to the complexity of the interfacial medium, both in terms of structural heterogeneity and dynamics (White and Wimley, 1998).

As shown in a previous work (Beswick et al., 1998a), PMP1, a small plasma membrane protein of the yeast *Saccharomyces cerevisiae* that regulates the activity of the H^+-ATPase (Navarre et al., 1992, 1994), should constitute a suitable simple model for exploring lipid-protein specificity. The PMP1 sequence is the following:

L-P-G-G-V^{5}-I-L-V-F-I^{10}-L-V-G-L-A^{15}-C-I-**A-I-I**^{20}-
A-T-I-I-Y^{25}**-R-K-W-Q-A**^{30}**-R-Q-R-G-L**^{35}**-Q-R-F**

PMP1 belongs to a class of small membrane proteins referred to as proteolipids (Folch-Pi and Stoffyn, 1972). These proteins exhibit a unique transmembrane hydrophobic segment followed by a highly charged domain. They function as regulatory subunits of membrane cation-transporting P-type ATPases. One of the most studied proteolipids is phospholamban, associated with the cardiac Ca^{2+}-ATPase (for a recent review see Simmerman and Jones, 1998).

The conformation of a synthetic fragment of PMP1 solubilized in perdeuterated dodecylphosphocholine micelles has been determined by ^{1}H-NMR (Beswick et al., 1998a). This fragment, referred as PMP1f in the following, spans the C-terminal region of PMP1, A_{18}-F_{38} (bold residues in the sequence aforementioned). It comprises a part of the hydrophobic segment and the whole cytoplasmic domain, whose five basic side chains are specifically distributed at the membrane interface and are thought to strongly interact with anionic phospholipids.

Solid-state ^{2}H-NMR spectroscopy enable the investigation of protein-membrane interactions (for a recent review see Watts, 1998) in multilamellar bilayer membranes, which avoid peptide-induced aggregation artifacts reported re-

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cently with unilamellar lipid vesicles (Murray et al., 1999). We are presenting an exhaustive ^{2}H-NMR study of the interactions between PMP1f and anionic phospholipids. For this purpose, selectively deuterated mixed POPC/POPS 5:1 multilamellar bilayers were used as membrane models. Spectra of headgroup- and chain-deuterated POPC and POPS phospholipids, POPC-d4, POPC-d31, POPS-d3, and POPS-d31, were recorded at different temperatures and for various concentrations of PMP1f.

MATERIALS AND METHODS

Peptide synthesis

The PMP1 fragment, PMP1f, was synthesized as previously reported in Beswick et al., 1998a.

Sample preparations

All lipids, including deuterated POPC-d4, POPC-d31, POPS-d3, and POPS-d31 were purchased from Avanti Polar Lipids (Alabaster, AL). POPC-d4 and POPS-d3 are deuterated at positions α and β of their respective headgroups. The palmitoyl chain of POPC-d31 and POPS-d31 is uniformly deuterated. POPS, POPS-d3, and POPS-d31 were under their sodium salt form. Liposomes were prepared by mixing lipid chloroform solutions in a PC/PS molar ratio of 5:1 and methanolic solutions of PMP1 fragment. The chosen PC/PS ratio constitutes a good compromise between a realistic proportion of PS in the membrane cytoplasmic leaflet and a satisfactory sensitivity for the PS deuteron signals. The solvent was then removed by evaporation under N_2. The solid residues were dried under vacuum (10^{-2} mm Hg) for 12 h and dispersed by continuous vortexing at 20°C in 100–500 μl of Tris buffer (50 mM in deuterium depleted water (Eurisotop, France) pH 7.0, 40 mM NaCl) giving ~100 mM lipid dispersions.

^{2}H-NMR experiments

^{2}H-NMR spectra were recorded at 46 MHz on a Bruker DMX 300 spectrometer equipped with a probe specifically designed for solid-state deuterium NMR experiments (Morris Ins., Canada). Spectra were acquired at different temperatures in a 0–37°C range with a dwell time of 2 μs, 4 K data points, and a recycling time of 50 or 200 ms for headgroup- or chain-deuterated lipids, respectively. The T_1 values of headgroup deuterons are small (~10 ms), so recycling delay of 50 ms can be used without significant loss of intensity. As a control experiment, spectra of headgroup-deuterated POPC and POPS recorded with recycling delay of either 50 or 500 ms were found to be superimposable. The larger recycling (200 ms) delay with chain-deuterated phospholipids is needed because of the larger T_1 (~50 ms) of the CH_2 groups located at the end of the lipid acyl chains (Davis, 1983). A quadrupolar echo pulse sequence (Davis et al., 1976) was used with a pulse length of 3 μs and pulse separation of 40 μs. Oriented ^{2}H-NMR spectra (0°) were obtained by the numerical De-Pake-ing procedure described by Sternin et al. (1983). The method of moments was applied to the chain deuteron spectra (Davis, 1979; Davis et al., 1979).

Building the PMP1 working model

The starting model was constructed by docking eight PS molecules against the NMR-derived structure of the PMP1 fragment. Low-temperature (50 K) dynamics and minimization were performed in vacuum for annealing the molecular ensemble and to obtain preliminary information on the interactions between lipid polar headgroups and peptide side chains. The calculations were done with the SYBYL Software (TRIPOS, Inc.).

RESULTS

Characterization of mixed POPC/POPS 5:1 model membranes

Before investigating the effect of PMP1f on POPC/POPS 5:1 membranes, we analyzed the behavior of these mixed membranes with the temperature, as reported by the ^{2}H-NMR spectra of POPC-d31/POPS and POPC/POPS-d31 samples (data not shown). We found that both the POPC-d31 and POPS-d31 signals conserve the characteristics of liquid crystalline phases between 37 and 10°C. We therefore performed our further experiments within a 10–37°C temperature range. Thus, our model membranes conserve the conditions of a homogeneous fluid phase, in good agreement with the available PC/PS phase diagrams (Silvius and Gagné, 1984) and previous ^{2}H-NMR data obtained with similar POPC/POPS 5:1 membranes (Roux and Bloom, 1990, 1991). ^{2}H-NMR experiments were then performed on selectively deuterated POPC/POPS 5:1 membranes containing increasing amounts of PMP1f. For the sake of clarity, the relative PMP1f concentration R is expressed with respect to the POPS concentration, i.e., R = [PMP1f]/[POPS]. Dividing the R value by 6 (5 PC + 1 PS) gives the peptide-to-total phospholipid (PL) concentration ratio, [PMP1f]/[PL].

PMP1f with POPC/POPS-d3 membranes

^{2}H-NMR spectra of membranes containing headgroup-deuterated POPS (POPS-d3) display three resolved quadrupolar splittings attributed to the α_1, α_2, and β deuterons of the serine moiety. As shown in Fig. 1, the largest quadrupolar splittings correspond to the α_1 and β deuterons, while the α_2 signal is overlapped by the residual water resonance (Roux and Bloom, 1990). Addition of PMP1f to POPC/POPS-d3 5:1 membranes induces both a progressive increase of the line-widths and the appearance of a very broad component. The resolved serine signals become hardly discernible at R = 0.3 and totally disappear when R reaches 0.5. The evolution of the POPS-d3 spectrum upon addition of PMP1f to POPC/POPS 5:1 membranes dramatically contrasts with that of POPC-d4 in identical mixed membranes, for which only a slight line-broadening is observed, as discussed below. The broad component reveals the existence of POPS molecules whose headgroup motions are severely restricted. Such a component associated with a progressive disappearance of the resolved POPS-d3 signals indicates the formation of specific PMP1f-POPS complexes

PMP1f with POPC/POPS-d31 membranes

Fig. 2 *A* shows the evolution of the ^{2}H-NMR powder spectrum of POPC/POPS-d31 5:1 membranes at 20°C upon

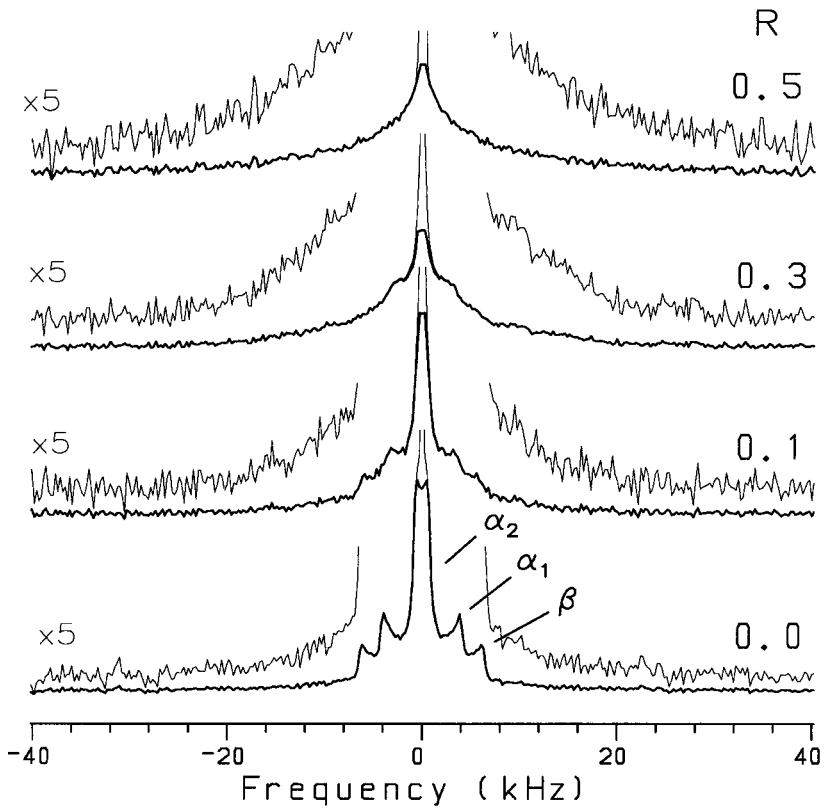

FIGURE 1 Effect of increasing amounts of PMP1f (relative concentration R = [PMP1f]/[POPS]) on the ^{2}H-NMR spectra of headgroup-deuterated POPS-d3 in POPC/POPS 5:1 bilayers at 20°C. The splittings corresponding to the three serine deuterons are indicated on the bottom spectrum. The central water peak was removed by subtracting a Lorentzian line before area-normalization of the spectra.

addition of PMP1f. On increasing the PMP1f concentration, a continuous broadening of the POPS-d31 signal is observed. Examination of the corresponding De-Paked spectra (Fig. 2 *B*) reveals the appearance of a second signal whose maximum quadrupolar splitting (31.5 kHz) is significantly greater than that observed for the PMP1f-free sample (29 kHz). On increasing the PMP1f concentration, the intensity of the second component increases at the expense of the PMP1f-free signal, which totally disappears at $R = 0.4$. The POPS-d31 De-Paked spectra clearly show the existence of a slow exchange at the ^{2}H-NMR time scale between two POPS species. In agreement with the POPS-d3 data, the evolution of the POPS-d31 spectrum is consistent with the formation of PMP1f-POPS complexes. From the difference observed between the maximum quadrupolar splittings of the two corresponding signals (2.5 kHz), an upper limit of 0.4 ms can be estimated for the exchange time between free and bound POPS molecules. When R is further increased up to 0.5 the spectrum becomes dramatically broadened, while the maximum quadrupolar splitting is severely reduced. Such a behavior indicates that a transition in the lipid-peptide organization of the complexes occurs when R exceeds 0.4. Analysis of this transition is out of the scope of this paper.

Considering that the De-Paked spectrum obtained at $R = 0.4$ corresponds to POPS molecules bound to PMP1f, the approximate ratio of peptide-bound to free POPS for different R values can be evaluated. This was achieved by estimating the proportion of the spectrum obtained at $R = 0.4$, present in the other spectra obtained at lower R values (Fig. 3 *A*). Conversely, we estimated the proportion of the spectrum corresponding to POPC/POPS-d31 membranes at $R = 0$, present in the spectra recorded after addition of PMP1f (Fig. 3 *B*). The two data sets obtained independently are in reasonable agreement. The amount of peptide-bound POPS, normalized with respect to the PMP1f concentration, was plotted versus the concentration ratio [POPS]/[PMP1f], i.e., $1/R$ (Fig. 4). A straightforward analysis of the resulting binding curve indicates that one PMP1f molecule can bind a maximum of 8 POPS molecules.

PMP1f with POPC-d31/POPS and POPC-d4/POPS membranes

In our previous paper devoted to PMP1f (Beswick et al., 1998a), we showed that, except for a slight broadening, the presence of PMP1f does not affect the POPC-d31 signals, both in pure POPC-d31 and mixed POPC-d31/POPS membranes. New experiments performed on the full 10–37°C range showed that there is no peptide-induced second component, and that the palmitoyl chain order parameters and the first and second moments of the powder patterns are similar for all samples with or without PMP1f (<10%

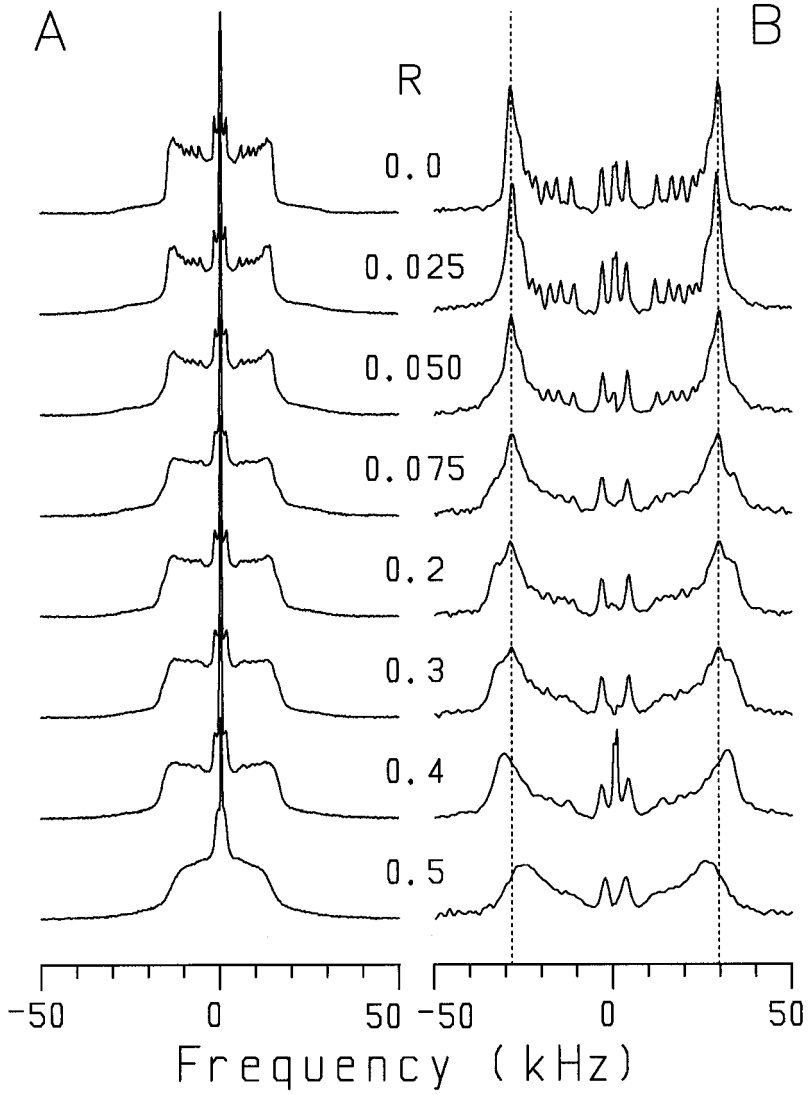

FIGURE 2 Effect of increasing amounts of PMP1f (relative concentration R = [PMP1f]/[POPS]) on the ^{2}H-NMR spectra of chain-deuterated POPS-d31 in POPC/POPS 5:1 bilayers at 20°C; (*A*) powder spectra, (*B*) De-Paked spectra. The central water peak was removed by subtracting a Lorentzian line before area-normalization of the spectra.

changes, data not shown). In contrast with the POPC-d31 signal, the PC headgroup α and β quadrupolar splittings ($\Delta\nu\alpha$ and $\Delta\nu\beta$) of POPC-d4/POPS membranes are sensitive to the addition of PMP1f, although no second component is observed. At low temperature and high PMP1f concentration, $\Delta\nu\alpha$ and $\Delta\nu\beta$ tend toward their values observed for pure POPC membranes. Given our results obtained with deuterated POPS, we thus performed an exhaustive set of experiments on POPC-d4/POPS membranes in order to more precisely monitor the formation of POPS-PMP1f complexes through the variations of $\Delta\nu\alpha$ and $\Delta\nu\beta$ of the choline headgroup.

Fig. 5 displays the quadrupolar splittings $\Delta\nu\alpha$ and $\Delta\nu\beta$ obtained for POPC-d4/POPS/PMP1f membranes containing increasing amounts of PMP1f and measured at different temperatures. On increasing the PMP1f concentration (R) at 10°C, $\Delta\nu\alpha$ decreases while $\Delta\nu\beta$ increases. The same holds for $\Delta\nu\alpha$ at all temperatures, but not for $\Delta\nu\beta$, which appears to be less and less sensitive to the peptide insertion when the temperature is increased. At 37°C the β quadrupolar splitting is quasi-constant. We can thus distinguish two limiting cases of the PMP1f-induced perturbations of POPC headgroups: 1) at low temperature, the PMP1f incorporation in POPC/POPS membranes leads to opposite changes of the $\Delta\nu\alpha$ and $\Delta\nu\beta$ quadrupolar splittings, one being increased, while the other is decreased, and 2) at high temperature, $\Delta\nu\alpha$ is decreased and $\Delta\nu\beta$ is not affected.

DISCUSSION

Observation of two separate components associated with bulk and protein-bound lipids is rather exceptional in solid-state NMR studies of membrane systems. There are nevertheless some examples (Jordi et al., 1990; Carbone and Macdonald, 1996; Saurel et al., 1998), all dealing with

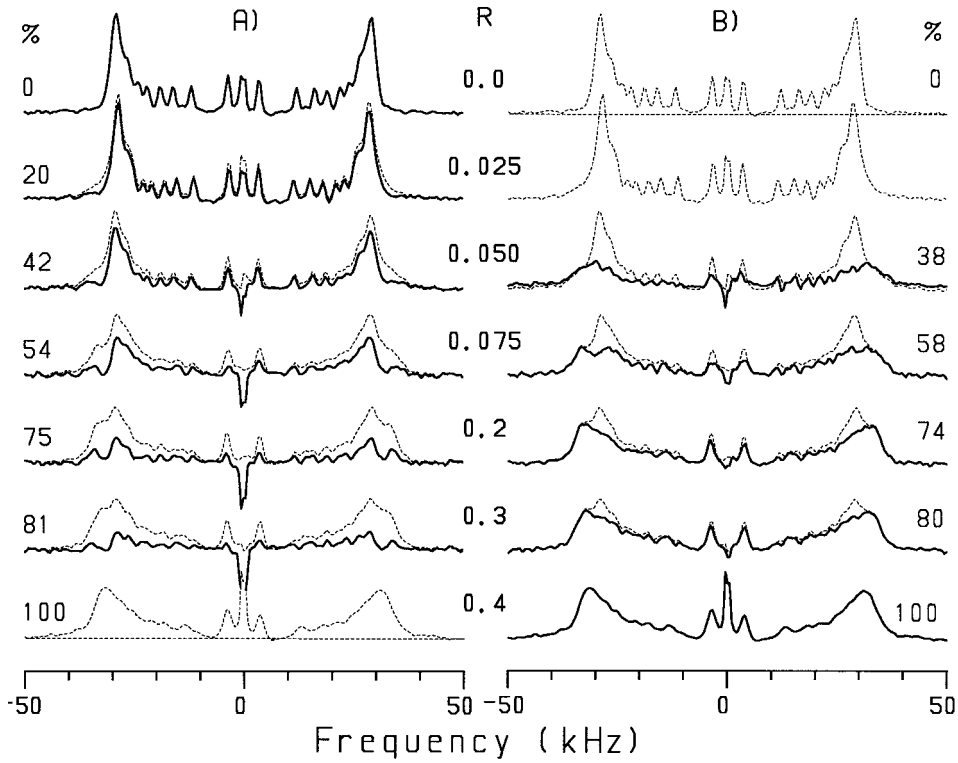

FIGURE 3 (*A*) Difference spectra (*bold trace*) obtained by subtracting appropriate fractions of the signal obtained at $R = 0.4$ from the spectra obtained at lower R values; the original spectra (Fig. 2 *B*) are shown as a dotted line. (*B*) Difference spectra obtained by subtracting fractions of the signal corresponding to POPC/POPS-d31 membranes at $R = 0$ from the spectra obtained in the presence of PMP1f. For the trace at $R = 0.025$, the subtraction was found unreliable.

mixed membranes comprising anionic phospholipids (phosphatidylserine, phosphatidylglycerol). Surprisingly, among the numerous deuterium NMR studies devoted to lipid-protein interactions (Watts, 1998), especially those concerning the specificity of anionic phospholipids, only a few used chain-deuterated phosphatidylserine (Devaux et al., 1986; Jordi et al., 1990; De Kroon et al., 1991; Saurel et al., 1998). However, they constitute a priori suitable probes in this context. In particular, the first deuterons of the PS acyl chains giving rise to the largest quadrupolar splittings ex-

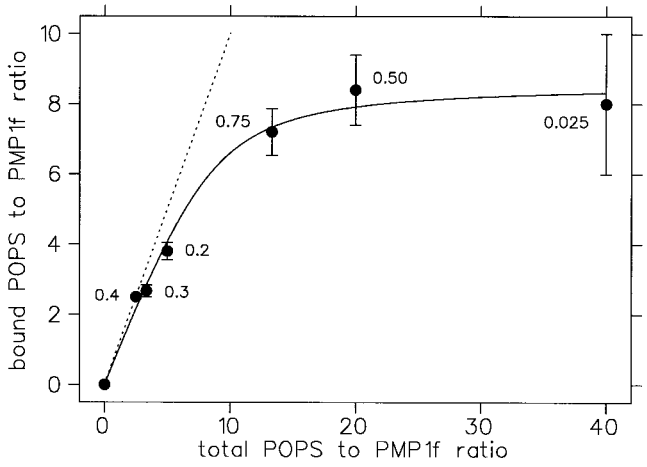

FIGURE 4 Binding of POPS to PMP1f in the lipid phase. The POPS concentrations are normalized relative to the PMP1f concentration in the lipid phase. The fractions of PMP1f-bound POPS are determined from the values listed in Fig. 3. The R values are indicated above each experimental data point. The incidence of the ±5% error made with the spectrum difference method used in Fig. 3 is shown with the error bars.

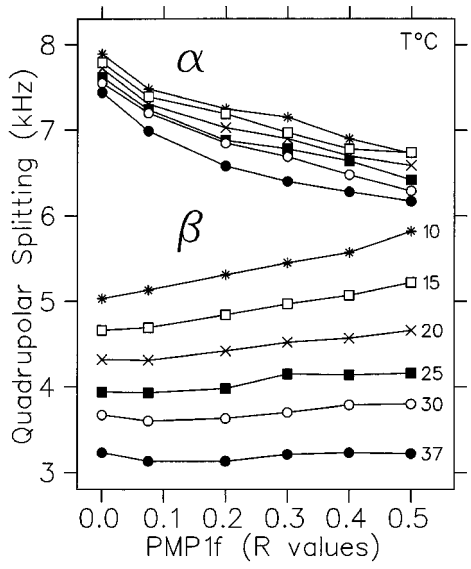

FIGURE 5 Plots of the POPC-d4 quadrupolar splittings (α and β) versus the relative concentration R = [PMP1f]/[POPS], obtained for POPC-d4/POPS 5:1 bilayers at different temperatures (experimental uncertainty, ±0.1 kHz). (●) 37°C, (○) 30°C, (■) 25°C, (×) 20°C, (□) 15°C, and (∗) 10°C.

perience more restricted segmental motions than other deuterons (except for those of the glycerol moiety). For given relative variations, the quadrupolar splittings of the acyl chains will give larger absolute variations than the small splittings of the polar headgroups, and should therefore exhibit an enhanced sensitivity to changes of the environment.

Stoichiometry of the PMP1f-POPS complex

The present work shows a favorable case where the large quadrupolar splitting of the POPS acyl chain plateau deuterons allows the separation of two spectral components, enabling the precise investigation of the POPS-PMP1f complexes. The data indicate that PMP1f is able to form a complex with about eight POPS molecules. We have already shown by ^{1}H-NMR that in a micellar environment, the peptide adopts a unique long helix conformation, extending from the N-terminus up to Q_{32} (Beswick et al., 1998a). The helix thus includes the hydrophobic segment A_{18}–I_{24}, which is embedded in the micelle hydrophobic core, and a part of the charged cytoplasmic domain, i.e., Y_{25}–Q_{32}. The amphipathic motif $Y_{25}RKW_{28}$ determines the interfacial location of the cytoplasmic domain. The helix is followed by a loop, R_{33}-F_{38}, whose C-terminal extremity folds back toward the micelle interior (Beswick et al., 1998b). The PMP1f conformation results in a crown-like interfacial distribution of the five basic and three glutamine side chain extremities (R_{26}, K_{27}, R_{31}, R_{33}, R_{37}, Q_{29}, Q_{32}, Q_{36}) around the helix. Such a distribution of basic side chains combined with the exceptionally high proportion of negatively charged lipids found in the cytoplasmic leaflet of the yeast plasma membrane (Zinser and Daum, 1995) suggests that the cytoplasmic domain of PMP1 could strongly interact with anionic phospholipids. Because the effective charge of the peptide is +5 (4 Arg, 1 Lys), a complex comprising about five POPS molecules would be expected. A stoichiometry of eight PS per PMP1f therefore suggests that the three glutamine residues, the remaining polar residues of the PMP1 cytoplasmic domain, also contribute to the lipid-peptide interactions. In fact, it is not necessary to interpret the stoichiometry of the complex as resulting from the number of interacting residues, but simply by considering the maximum accessibility offered to PS molecules by the peptide, given its conformation. Molecular modeling clearly shows that a maximum of about eight lipid molecules can be simultaneously in contact with the peptide area. A working model of a complex of PMP1f with eight PS molecules obtained by a simple docking procedure in vacuum and energy minimization indicates that the Arg, Lys, and Gln side chains readily form a wide hydrogen bond network with the phosphate and carboxylate groups of PS headgroups. Within this network, an Arg side chain can form salt bridges with two PS negatively charged groups. Such electrostatic interactions most probably constitute the main long-range driving forces responsible for the PMP1-PS complex formation. However, our preliminary model indicates that the glutamine side chains, acting both as H-bond donor and acceptor groups, contribute efficiently to the stability of the PMP1-PS complex.

PMP1f-induced electrostatic perturbations of POPC headgroups

The incorporation of positively charged PMP1f in POPC/POPS 5:1 is expected to change the electrical properties of the membrane surface. Since the pioneering work of J. Seelig (Brown and Seelig, 1977; Akutsu and Seelig, 1981), it has been well known that the phosphatidylcholine α and β deuteron signals constitute suitable parameters for probing membrane electrical perturbation in liquid crystalline phases, through the "molecular voltmeter effect" of the choline headgroup (Seelig et al., 1987).

When positive ions or positively charged polypeptides are added to zwitterionic or negatively charged membranes, the conformational response of PC headgroups is characterized by opposite variations of the choline quadrupolar splittings: $\Delta\nu\alpha$ decrease and $\Delta\nu\beta$ increase. Another type of "conformational response" is associated with the increase of the specific volume occupied by the choline headgroup. This response, characterized by a reduction of the β splitting, can simply result from a temperature increase. It can be viewed in Fig. 5 with the data obtained at a given peptide concentration. For instance, reducing the temperature from 37°C to 10°C in the absence of peptide ($R = 0$), leads to a large increase of $\Delta\nu\beta$ ($\sim$+60%), while $\Delta\nu\alpha$ is barely affected ($\sim$+6%). Variations of $\Delta\nu\beta$ can also be observed at a fixed temperature, in the presence of molecules such as cholesterol or chloroform, acting as a "spacer" between PC molecules (Brown and Seelig, 1978; Akutsu and Seelig, 1981). These particular perturbations, which specifically change the value of $\Delta\nu\beta$, and which we will refer to as the "spacer" effect in the following, are probably related to a weakening, or even the suppression in the case of cholesterol, of the intermolecular interactions between adjacent choline headgroups (Brown and Seelig, 1978). Indeed, the "spacer" effect counteracts the electrical effect induced on $\Delta\nu\beta$ by positively charged molecules when both types of perturbation take place. We believe that the PMP1f-induced variations of the α and β quadrupolar splittings reported in this paper can be rationalized by a combination of the electrical and "spacer" conformational response. At 10°C a nearly pure electrical effect is observed, reflecting the change of the membrane surface charge due to the increasing amounts of positively charged PMP1f. Between 15 and 37°C, when R increases, the *slope* of the $\Delta\nu\beta$ versus R plot progressively decreases on increasing the temperature. This

could be related to the occurrence of a PMP1f-induced "spacer" effect counteracting the electrical effect on $\Delta\nu\beta$, and attenuating the charge-induced increase of the β quadrupolar splitting. We propose that this "spacer" effect is associated with a perturbation of the POPC headgroups in contact with the POPS/peptide complexes. The fact that the "spacer" effect is barely observed at 10°C could simply indicate that the later complexes laterally segregate at low temperature, excluding the PC molecules from the PMP1f-POPS environment and allowing intermolecular interactions between PC headgroups. Conversely, on reheating the sample, the PMP1f-POPS aggregates would partially dissociate and surround some PC molecules, hindering intermolecular interactions between PC headgroups. At 37°C, the slope of the $\Delta\nu\beta$ versus R plot is null, which means that the "spacer" effect fully offsets the electrostatic response.

CONCLUSION

The data obtained with chain-deuterated POPS indicate that the PMP1f-POPS interactions are strong enough to induce the formation of a molecular complex. The choline response obtained in the presence of PMP1f could indicate that they also lead to a lateral segregation of these complexes in the bilayer.

Our previous ^{1}H-NMR study of PMP1f (Beswick et al., 1998a, b) and the present ^{2}H-NMR results obtained with the PMP1f-POPC/POPS system, as well as the small size of PMP1, provide a suitable model of protein interactions with lipid bilayers. We thus have undertaken a molecular dynamics simulation on a hydrated PC/PS bilayer including PMP1. The result of this simulation should provide a detailed description of the interaction network that takes place between PMP1 and phosphatidylserines. In order to complete this theoretical approach, ^{1}H and ^{2}H-NMR studies of PMP1f mutants are also underway.

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